In order to expand the regime of applications to high-resolution colocalization studies we designed a setup which allows for STED imaging of up to three different labels (quasi-)simultaneously. This enables us to accurately investigate the interplay of at least three differently labeled cellular components [1]. We distinguish the labels by using different spectral properties of the dyes and, in addition, by separating dyes with similar spectral properties but different fluorescence lifetimes. The design intrinsically provides high spatial resolution for all markers simultaneously, since in STED, the diffraction barrier is overcome by reducing the effective volume in which fluorescence can occur spontaneously to a nanosized region at the very center of the STED beam. Our approach is less prone to drift artifacts than e.g. stochastic imaging concepts where the recording of even a small region of the object extends over the whole image acquisition period, and it is not subject to systematic errors due to the fluorophore orientations which have to be considered in stochastically based high-resolution techniques.

[1] D. Neumann, J. Bückers, L. Kastrup, S. W. Hell, S. Jakobs: "Two-color STED microscopy reveals different degrees of colocalization between hexokinase-I and the three human VDAC isoforms," PMC Biophysics, **3**:4 (2010).

1920-Plat

3D Single Molecule Tracking in Thick Cellular Samples using Multifocal Plane Microscopy

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Protein transport across a cell monolayer represents a fundamental cellular process in cell biology. However, the imaging of this process poses several challenges. Protein transport is highly dynamic occurring across the entire cell-monolayer, which is several tens of microns thick, and over widely varying time scales. Additional challenges arise if 3D single molecule tracking is also desired that are particularly related to imaging single molecules for extended periods of time and across varying depths in a thick sample. Approaches that use a focusing device are not well suited for such applications as they are typically slow and suffer from poor z-localization/3D-tracking capability. To date the 3D spatial-temporal dynamics of many biological processes are poorly understood, as there is a lack of an appropriate methodology that supports 3D single-molecule tracking in thick samples.

Here we show that multifocal plane microscopy (MUM) [1-2] provides the much needed solution to this longstanding problem. While MUM has been previously used for 3D single molecule tracking across shallow depths (1 micron) in live cells [2], the question arises if MUM can also live up to the significant challenge of tracking single molecules in a thick sample. By substantially expanding the capabilities of MUM, here we demonstrate 3D tracking of quantum-dot labeled single molecules in a ~10 micron thick live-cell monolayer, for extended periods of time (minutes). We have implemented a 4-plane MUM setup with increased focal plane spacing and a novel multicolor imaging approach, which enables the imaging of the molecule of interest as well as the cellular environment with which it interacts. Using this new methodology we have reconstructed the complete 3D trafficking itinerary of single molecules at high spatial and temporal resolution.

1. PNAS, 2007, 104:5889-5894.
2. Biophys J., 2008, 95:6025-6043.

1921-Plat

Picosecond Spectral Cars Imaging with Principal Component Analysis

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Imaging methods based on vibrational Raman contrast offer label-free chemical imaging of tissues. Video rate imaging acquisition times can be achieved by coherent Raman techniques, however, they do so only at a single vibrational frequency - limiting a more in-depth chemical analysis of the tissue. The most direct solution is to extend the measurement in the vibrational frequency domain, which has been manifested in the development of broadband coherent Raman methods. Unfortunately, the attributed acquisition time renders them to be impractical in examining large tissue areas. In this study, we demonstrate that picosecond spectral coherent anti-Stokes Raman scattering (CARS) imaging combined with principal component analysis (PCA) can be utilized to achieve fast high-resolution maps of lipids in tissues. Spectral CARS data sets were generated by imaging tissue sections at consecutive Raman shifts in the CH_2 stretching vibrational range. By avoiding spectral redundancy, the acquisition time was shortened, whereas loss of spectral information was compensated by a much higher information density in the spatial domain via PCA. This resulted in sufficient spectral contrast, which was illustrated in our recent application in characterizing lipids distribution and composition of the meibomian glands in the context of dry eye disease. Spectral differences between CARS spectra obtained from specific regions were confirmed with a Raman microspectroscopic analysis. The resulting multi-component chemical maps revealed a lipid maturation process throughout the gland structure, which was not observed before and may provide important clues toward understanding meibomian gland dysfunction.

1922-Plat

Fast STED Microscopy for Live Cell Imaging

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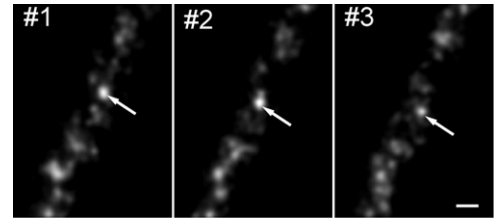
We demonstrate Fast Stimulated Emission Depletion (STED) microscopy for live cell imaging. The suitability of pulsed and cw lasers is shown.

Imaging with a spatial resolution of ~60 nm and a temporal resolution of 36 ms (28 frames per second) was used for a detailed analysis of the motion of synaptic vesicles in living neurons.

Automated data analysis via image processing and single particle tracking was used for quantifying the motion behavior. We found that newly endocytosed vesicles are highly mobile, for substantial time after endocytosis. They later undergo a maturation process, integrating into clusters, where they exhibit little mobility. After exocytosis, the vesicle material is temporarily on the plasma membrane; the mobility of such vesicle components increased when higher quantities of vesicle material were fused onto the plasma membrane.

Fast STED microscopy allows high-resolution imaging with up to 200 frames per second, which is exemplified by the observation of colloidal-crystal formation.



Our studies demonstrate the emerging ability of optical microscopy to investigate not only inanimate but also intracellular physiological processes on the nanoscale in real-time. The figure shows the movement of synaptic vesicles. Three frames of a 30s movie. Scale bar 250nm.

1923-Plat

Super-Resolution Microscopy Reveals Protein Spatial Reorganization in Early Innate Immune Responses

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Over the past decade optical approaches were introduced that effectively break the diffraction barrier. Of particular note were introductions of Stimulated Emission/Depletion (STED) microscopy, Photo-Activated Localization Microscopy (PALM), and the closely related Stochastic Optical Reconstruction Microscopy (STORM). STORM represents an attractive method for researchers, as it does not require highly specialized optical setups, can be implemented using commercially available dyes, and is more easily amenable to multicolor imaging. We implemented a simultaneous dual-color, direct-STORM imaging system through the use of an objective-based TIRF microscope and filter-based image splitter. This system allows for excitation and detection of two fluorophors simultaneously, via projection of each fluorophor's signal onto separate regions of a detector. We imaged the sub-resolution organization of the TLR4 receptor, a key mediator of innate immune response, after challenge with lipopolysaccharide (LPS), a bacteria-specific antigen. While distinct forms of LPS have evolved among various bacteria, only some LPS variations (such as that derived from E. coli) typically result in significant cellular immune response. Others (such as from the plague bacteria Y. pestis) do not, despite affinity to TLR4. We will show that challenge with LPS antigens produces a statistically significant increase in TLR4 receptor clusters on the cell membrane, presumably due to recruitment of receptors to lipid rafts. These changes, however, are only detectable below the diffraction limit and are not evident using conventional imaging methods. Furthermore, we will compare the spatiotemporal behavior of TLR4 receptors in response to different LPS chemotypes in order to elucidate possible routes by which pathogens such